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Specific Reversal of Cytolytic T Lymphocyte–Target Cell Interaction

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A functional assay is described that measures the reversal of specific cytolytic T cell (CTL)-target cell binding. Binding of ^{51}Cr-labeled P815 cells was stable in suspension but could be readily reversed by the addition of unlabeled P815 cells. The reversal of CTL-tumor cell and CTL-spleen cell binding was H-2 specific; only cells of the same H-2 type as the bound target cell could induce reversal. In all cases, tumor cells were substantially more efficient than spleen cells in inducing specific reversal.

Key words: cell recognition, reversal of cell interaction, target cell interaction, cytolytic T lymphocytes, H-2 recognition, reversal, cell adhesion

The understanding of cell recognition by cytolytic T lymphocytes (CTL) has developed somewhat differently from that in other cell-cell recognition systems. The use of inbred mouse strains has allowed investigators to demonstrate that molecules encoded in the major histocompatibility complex (MHC, in the mouse, H-2) and expressed by the target cell are required for recognition by CTL. The genetic approach and the use of specific antibodies against target cell surface proteins have allowed the identification of H-2K and H-2D cell surface glycoproteins as the target antigens for recognition by CTL [1]. CTL generated by stimulation with allogeneic cells (cells differing in the H-2 complex) appear to recognize polymorphisms in these homologous H-2 molecules. CTL generated against modified syngeneic cells (virus-infected or chemically modified cells, or cells that differ in minor histocompatibility loci) recognize the modifying antigen(s) only when present on cells bearing the syngeneic H-2K or D molecules. This observation, termed H-2 restricted recognition, has led to the proposition that CTL either possess two receptors (one for self-H-2 and one for the modifying antigen) or that CTL recognize a complex formed between self-H-2 and the modifying antigen [2].

The study of CTL recognition at the molecular level has been hindered by great difficulties in demonstrating CTL recognition of subcellular material derived from target cells. Attempts to block CTL-mediated lysis of target cells with purified target cell plasma membrane preparations have, in general, been unsuccessful.

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In one successful report the specificity was not impressive, and blocking activity was seen only with some membrane preparations [3]. One report of CTL-mediated lysis of liposomes bearing target cell proteins has appeared. Here, too, the activity was seen only in some preparations [4]. In marked contrast to these difficulties, purified plasma membranes and liposomes containing purified target cell surface proteins have been used successfully to stimulate the generation of specific allogeneic CTL from pre CTL [5, 6; see Mescher and Herrmann, this volume].

The first step in CTL-mediated lysis is specific binding of the target cell, to form stable CTL–target cell conjugates. In the presence of Ca^{++} the CTL then delivers a lethal hit, which results in eventual target cell lysis [7]. CTL activity is generally assayed by measuring the rate of ^{51}Cr release from labeled target cells after the CTL and target cells have been copelleted. In an attempt to facilitate CTL interaction with purified plasma membranes or liposomes, we have developed an assay that measures CTL binding of target cells in suspension [8]. Using this assay we have shown that CTL binding of target cells has the appearance of an equilibrium binding process. Scatchard plots could be used to derive relative affinities of CTL for target cells. CTL generated by stimulation with purified membranes were found to have a higher affinity for target cells and to be fewer in number than CTL generated by stimulation with intact cells. Membranes could not, however, specifically block target cell binding in suspension [8].

Investigation of CTL–target conjugate formation in suspension led to the finding that these conjugates can be readily reversed by interaction with free target cells [9]. CTL appear to provide a useful model system for studying reversal of specific cell-cell interactions. This report describes the results of experiments done to investigate the requirements for this reversal of CTL–target cell binding.

MATERIALS AND METHODS

Mice and Tumor Cells

C57BL/6 (B6, H-2^b) and AKR (H-2^k) were from Jackson Laboratory, Bar Harbor, Maine. (BALB/c × DBA/2) F1 (CD2, H-2^d) were from Cumberland View Farms, Clinton, Tennessee. P815 (H-2^d), a mastocytoma of DBA/2 origin, was maintained by passage in ascites in CD2 mice. EL4 (H-2^b), a B6 thymoma, was passaged in ascites in B6 mice.

CTL and Target Cell Preparation

Spleen cells from B6 mice immunized with 1×10^7 P815 IP were used as primed anti-H-2^d responder cells. CD2 mice immunized with 2×10^7 EL4 IP were used as primed anti-H-2^b responders. CTL were generated by secondary in vitro stimulation of primed spleen cells with irradiated, allogeneic spleen cells [8]. Five $\times 10^7$ primed spleen responder cells from animals immunized at least 1 month previously were cultured with 2×10^7 irradiated allogeneic spleen stimulator cells in 20 ml of supplemented RPMI 1640 (GIBCO, Grand Island, New York) medium [8]. Cultures were harvested after 4–6 days and CTL were enriched for on Ficoll-Hypaque gradients (Pharmacia, Piscataway, New Jersey). P815 target cells were prepared by incubating 2×10^7 P815 in 1 ml RHS medium [8] with 0.3 mCi ^{51}Cr ($Na^{51}CrO_4$, 1 mCi/ml, New England Nuclear, Boston) for 1 hour at 37°C. For spleen cell targets, cells teased from one spleen were incubated in 1 ml RHS medi-

um plus 0.3 mCi ^{51}Cr for 1.5 hours at 37°C. Red blood cells were then removed on a Ficoll-Hypaque gradient. Unlabeled spleen cells were also Ficoll-Hypaque purified. All cells were washed several times in RHS medium, resuspended in EGTA/Mg^{++} medium (see below) and counted.

CTL Assay

RHS medium is RPMI 1640 without sodium bicarbonate (GIBCO) supplemented to give the following final concentrations: 10 mM HEPES (N-2-hydroxyethyl piperazine-N′-2-ethane sulfonic acid) (Sigma, St. Louis), pH 7.3, 127 mM NaCl, 1.8 mM $MgCl_2$, 1.3 mM $CaCl_2$, 50 U/ml penicillin, 50 μg/ml streptomycin, 10% heat-inactivated calf serum (GIBCO) and 2 mM added glutamine. EGTA/Mg^{++} medium is RHS with 5mM EGTA (ethylene glycol-bis(β-aminoethyl ether) N, N′-tetra acetic acid) (Sigma) and 3.5 mM $MgCl_2$. Dextran/Ca^{++} medium is RHS with 10% (w/v) high molecular weight dextran (dextran T-500, Pharmacia) [8]. To form CTL–target cell conjugates in suspension, cells in 0.3 ml EGTA/Mg^{++} medium in 12 × 75 mm plastic round bottom tubes (Sarstedt, Princeton, New Jersey) were kept in suspension on an orbital shaker (25 cm platform, Bellco Glass, Vineland, New Jersey) at a setting of 3.5–4.0 [8]. Conjugate formation by centrifugation was done in 12 × 75 mm plastic conical tubes (Sarstedt) by centrifuging for 5 min at 250g followed by resuspension [9]. For the reversal assay, CTL–target conjugates were mixed with EGTA/Mg^{++} medium containing varying numbers of unlabeled cells. Reversal was then carried out either in suspension or by centrifugation as described above [9]. The number of functional CTL-labeled target cell conjugates present at any given time was determined by mixing $\leq$ 100μl of cells with 2 ml of dextran/Ca^{++} medium (final EGTA concentration $\leq$ 0.25 mM). The cells were then incubated for 3.5–5 hr at 37°C for ^{51}Cr-labeled P815 targets and at 29°C for labeled spleen cell targets, and the amount of specific ^{51}Cr release was determined [8]. This lower temperature for spleen cell targets yields a much lower spontaneous release of ^{51}Cr without decreasing the specific release.

RESULTS

The assay used to measure reversal of functional CTL–target cell binding is outlined in Figure 1. Target cell binding is Mg^{++}-dependent [10] and relatively temperature-independent [11, 12]. Delivery of the lethal hit requires Ca^{++} [10, 13] and is faster at 37°C [7, 11, 12]. CTL and ^{51}Cr-labeled target cells are therefore mixed in medium containing 5 mM EGTA at ambient temperature (about 23°C); these conditions allow target cell binding while very effectively preventing lysis of bound target cells [8]. CTL–target cell conjugates are allowed to form in suspension for several hours or they are formed rapidly by centrifugation. The cells are then diluted, and varying concentrations of unlabeled targets are added. The ability of these unlabeled cells to reverse the preformed CTL–^{51}Cr-labeled target cell conjugates is then tested in suspension or by centrifugation. The number of functional CTL–target cell conjugates remaining after varying times in suspension or after centrifugation is determined by adding small aliquots of cells to dextran/Ca^{++} medium and shifting to 37°C. The Ca^{++} allows lysis of bound target cells, whereas the viscous, high molecular weight dextran medium prevents CTL interaction with new target cells [8].

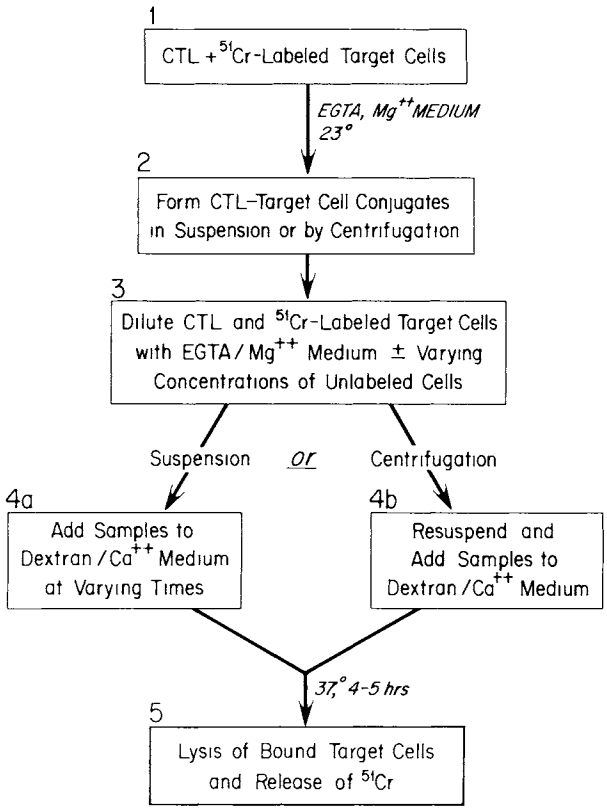

Fig. 1. Procedure for measuring reversal of functional CTL-target cell conjugates.

We have shown that target cell binding in suspension by CTL appears to be an equilibrium-binding process [8]. The stability of CTL–target cell conjugates was therefore investigated by forming conjugates at high cell concentrations and then diluting to very low concentrations. When CTL–P815 conjugates were formed by centrifugation and then placed in suspension, no significant reversal of target cell binding was seen (Fig. 2A, upper curve). In this same experiment an equivalent number of CTL and P815 target cells (1×10^5 P815 + 6×10^5 CTL per ml) were allowed to form conjugates in suspension; the target cell binding in this case reached a plateau level of only about 5% (Fig. 2B, lower curve). This plateau level of binding is about 10-fold lower than the level maintained by the centrifuged CTL, demonstrating that the rate at which CTL–P815 conjugates reverse spontaneously is extremely slow. The reverse rate is markedly enhanced, however, by the addition of unlabeled P815 target cells (Fig. 2A). The addition of ^{51}Cr-labeled target cells does not enhance the reverse rate (data not shown). This suggests that the reversal observed with unlabeled P815 is due to exchange of bound, labeled P815 for unlabeled P815. This reversal process is specific in that other, non-H-2^d tumor cell lines do not affect the reverse rate [9].

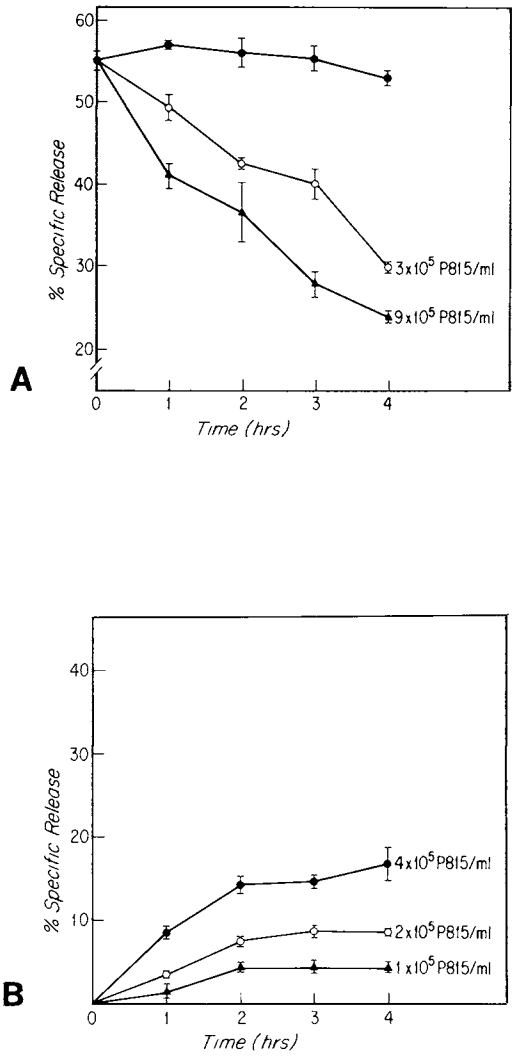

Fig. 2. Formation and reversal of CTL-P815 conjugates in suspension. CTL and ^{51}Cr-labeled P815 target cells were mixed in EGTA/Mg^{++} medium at a constant effector-to-target ratio (E/T) of 6:1. A) Target cells at 1.5 × 10^5/ml and CTL at 9 × 10^5 /ml were centrifuged to form conjugates. Aliquots of 0.2 ml were added to 0.1 ml of EGTA/Mg^{++} medium containing varying numbers of unlabeled P815 and were kept in suspension for 1–4 hours. The final concentration of ^{51}Cr-labeled P815 was 1 × 10^5/ml and unlabeled P815 were: none (●), 3 × 10^5/ml (○), or 9 × 10^5/ml (▲). B) Labeled P815 target cells at 4 × 10^5/ml (●), 2 × 10^5/ml (○), or 1 × 10^5/ml (▲) were mixed with CTL (at a constant E/T of 6:1) in 0.3ml of EGTA/Mg^{++} medium. The cells were kept in suspension without a prior centrifugation, and the rate of conjugate formation was measured. All points are the average ± 1 SEM (standard error of the mean) of quadruplicate samples. The spontaneous release (after 1–4 hours in suspension plus 4¼ hours in dextran/Ca^{++} medium) from target cells in the absence of CTL was 10.3–11.5%.

Similar results were obtained when the initial CTL–P815 conjugates were formed in suspension at a high cell concentration and then diluted (Fig. 3). After 1 hour approximately 20% of the CTL-labeled P815 conjugates were reversed at 8 × 10^6 unlabeled P815/ml, with less reversal occurring at the lower concentration. By way of comparison, using 7 mM EDTA to remove the Mg^{++} required for CTL–target cell binding resulted in reversal of 90% of these conjugates in 1 hour (not shown). Spleen cells of the correct H-2 type (H-2^d) were not effective in inducing reversal of these CTL–P815 conjugates (Fig. 3).

Spleen cells can serve as target cells for CTL-mediated lysis, but several lines of evidence suggest that spleen cell binding by CTL is weaker relative to P815. CTL-mediated lysis of spleen cell targets does not occur in suspension, whereas lysis of P815 and LPS (lipopolysaccharide) blast cells proceeds efficiently in suspension [8]. Tighter or more rapid binding of target cells is apparently required in suspension to resist the shear forces created. We have also observed that CTL–spleen cell conjugates (formed by centrifuging CTL with ^{51}Cr-labeled spleen cells) are not stable in suspension [9]. Finally, large numbers of unlabeled H-2^d spleen cells are required to block CTL-mediated lysis of P815 in standard cold target inhibition experiments [14].

We therefore tested whether H-2^d spleen cells would reverse CTL–P815 conjugates after centrifugation (Fig. 4). CTL–P815 conjugates were formed by centrifugation. They were then resuspended, and varying numbers of unlabeled spleen

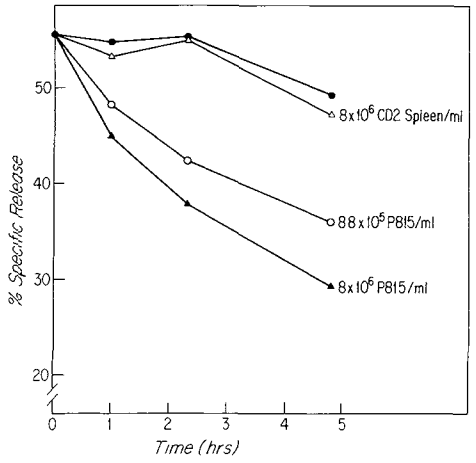

Fig. 3. Reversal of CTL–P815 conjugates by P815 and spleen cells. CTL were generated by secondary in vitro stimulation with P815 membranes [8]. CTL and ^{51}Cr-labeled P815 were mixed in 0.3ml EGTA/Mg^{++} medium at 1.3 × 10^6/ml and 8 × 10^6/ml, respectively. Conjugates were formed in suspension for 4⅓ hours and then diluted. One-tenth milliliter of the diluted conjugates was added to 0.2 ml EGTA/Mg^{++} medium containing unlabeled cells. The final dilution of conjugates was 20-fold to 4 × 10^5 labeled P815/ml. The final concentrations of unlabeled cells were: non (●), 8.8 × 10^5 P815/ml (○), 8 × 10^6 P815/ml (▲), or 8 × 10^6 CD2 spleen cells/ml (△). Reversal was carried out in suspension for 1–5 hours followed by 5 hours in dextran/Ca^{++} medium. The spontaneous release in the absence of CTL was 16–26%. All points are the average of triplicate samples; the SEM was below 1.5% for all points.

cells or tumor cells were added. The cells were then centrifuged under the same conditions used to form the initial CTL-P815 conjugates (250g for 5 min at 23°C). The cell pellets were allowed to sit for another 8 min at 23°C and were finally resuspended and added to dextran/Ca^{++} medium. Unlabeled P815 reversed very effectively, but EL4 (H-2^b) tumor cells were ineffective. The CD2 (H-2^d) spleen cells had only a slight specific effect.

From the above experiments it was unclear whether spleen cells were intrinsically inefficient in reversing CTL-target cell conjugates, or whether their ineffectiveness was dependent on the particular ^{51}Cr-labeled target cell used. Reversal of CTL-spleen cell conjugates was therefore examined. CTL and ^{51}Cr-labeled CD2 spleen cells were centrifuged to form CTL-spleen cell conjugates. The ability of various unlabeled cells to reverse these conjugates by centrifugation was then tested (Fig. 5). In this case unlabeled CD2 spleen cells yielded a very significant level of reversal. The unlabeled P815 were still far more efficient, however.

To test whether the very efficient reversal seen with P815 is due to a nonspecific effect, we examined CTL generated against another H-2 type. Anti-H-2^b CTL were mixed with ^{51}Cr-labeled B6 (H-2^b) spleen cells, and conjugates were formed by centrifugation. These conjugates were reversed by unlabeled EL4 (H-2^b) tumor cells and B6 spleen cells, but not by P815 (H-2^d) (Fig. 6). It is apparent that in this case also the EL4 tumor cells were substantially more effective than the B6 spleen cells.

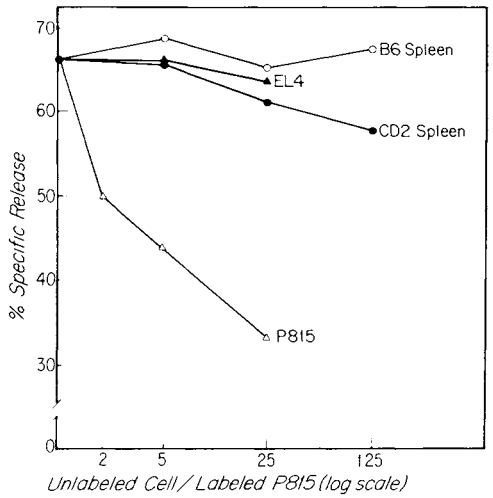

Fig. 4. Reversal of CTL-P815 conjugates by centrifugation. Conjugates were formed by centrifuging 1.5 × 10^6 CTL with 2 × 10^5 labeled P815 in 0.8ml of EGTA/Mg^{++} medium. Aliquots of 0.2 ml were then added to 0.1 ml EGTA/Mg^{++} medium containing unlabeled cells, and were centrifuged again as above (5 min at 250g). The cell pellets sat an additional 8 minutes at 23°C and were then added to dextran/Ca^{++} medium for 4 hours. The unlabeled cells were B6 spleen cells (○), CD2 spleen cells (●), EL4 (▲), or P815 (△). The final concentration of labeled P815 was 1.3 × 10^5/ml; the ratio of unlabeled cells to labeled cells is shown. All points are the mean of quadruplicate samples; the SEM was below 2% for all points. Spontaneous release in the absence of CTL was 10.3%.

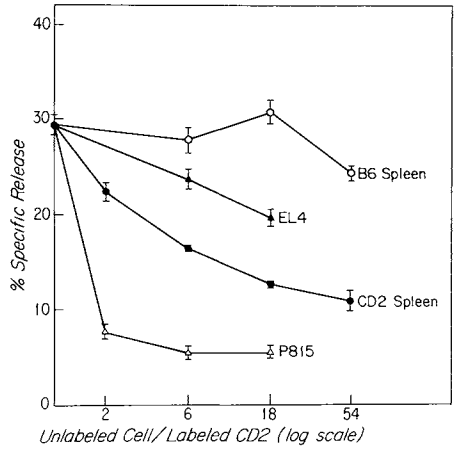

Fig. 5. Reversal of CTL–CD2 spleen cell conjugates. Conjugates were formed by centrifuging 1.9 × 10^6 CTL/ml with 3 × 10^5 labeled CD2 spleen cells/ml in 0.6 ml EGTA/Mg^{++} medium. Aliquots of 0.2 ml were then added to 0.1 ml EGTA/Mg^{++} medium containing unlabeled cells, and the cells were centrifuged again as above. After sitting an additional 6 minutes the cells were resuspended and added to dextran/Ca^{++} medium for 4.5 hours at 29°C. The unlabeled cells were B6 spleen cells (○), EL4 (▲), CD2 spleen cells (●), or P815 (△). The final concentration of ^{51}Cr-labeled CD2 spleen cells was 2 × 10^5/ml; the ratio of unlabeled to labeled cells is indicated. All points are the mean ± 1 SEM of quadruplicate samples. The spontaneous release in the absence of CTL was 11.1%.

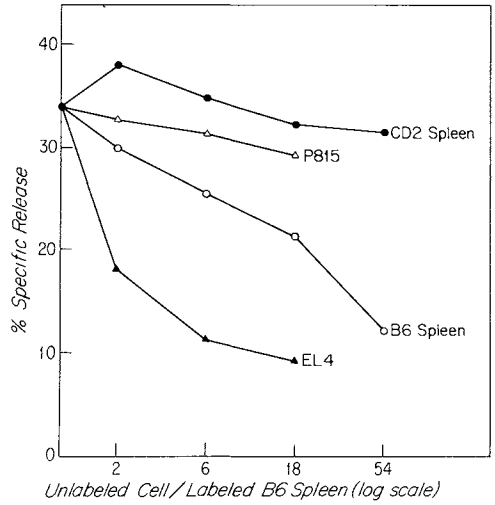

Fig. 6. Reversal of CTL–B6 spleen cell conjugates. Conjugates were formed by centrifuging 3 × 10^6 anti-H-2^b CTL/ml with 3.75 × 10^5 labeled B6 spleen cells/ml in 0.6 ml EGTA/Mg^{++} medium. Aliquots of 0.2 ml were then added to 0.1 ml of EGTA/Mg^{++} medium containing unlabeled cells, and the cells were centrifuged again. The cells were resuspended after sitting for an additional 5 min and were added to dextran/Ca^{++} medium for 4¼ hours at 29°C. The unlabeled cells were CD2 spleen cells (●), P815 (△), B6 spleen cells (○), or EL4 (▲). The final concentration of ^{51}Cr-labeled B6 spleen cells was 2.5 × 10^5/ml; the ratio of unlabeled to labeled cells is indicated. All points are the average of quadruplicate samples; the SEM averaged 1.3%, with 2.2% being the highest value. The spontaneous release in the absence of CTL was 22.9–29.5%.

These results demonstrate that a specific interaction with free target cells induces reversal of CTL–target cell conjugates. We have not been able to demonstrate specific reversal by purified plasma membranes from P815 cells [9 and unpublished data]. It is unclear what characteristic(s) of intact P815 cells are responsible for their preferential interaction with CTL, compared with spleen cells or purified membranes. The effect of glutaraldehyde fixation on recognition by CTL has been examined by Bubbers and Henney [15]. They found that P815 cells treated with 0.15% glutaraldehyde for 10 sec lost their ability to synthesize protein or nucleic acid, but could still be lysed by CTL. We have similarly found that mild glutaraldehyde fixation (up to 0.3% for 1 min) does not destroy the cells' ability to reverse CTL–P815 conjugates (Table I). At higher concentrations of glutaraldehyde during fixation the cells become ineffective.

DISCUSSION

We have shown in this report that CTL binding of target cells can be specifically reversed by interaction with free target cells. This reversal, measured functionally as a decrease in the amount of ^{51}Cr released from preformed CTL–target cell conjugates, presumably reflects physical release of bound, ^{51}Cr-labeled target cells. The ability of free target cells to induce reversal is dependent upon their H-2 type. Reversal therefore appears to have the same H-2 specificity as does target cell binding. Substantial differences were seen, however, in the ability of H-2 identical cells to induce reversal. Spleen cells effectively reversed CTL–spleen cell conjugates (Figs. 5, 6) but not CTL–P815 conjugates (Figs. 3, 4). P815 cells, in contrast, very efficiently reversed both types of conjugates (Figs. 2–5). Both P815 and EL4 cells were substantially more efficient than their H-2 identical spleen cells in reversing CTL–spleen cell conjugates (Figs. 5, 6).

TABLE I. Reversal of CTL–P815 Conjugates by Glutaraldehyde-Fixed P815 Cells

Glutaraldehyde concentration	Fixation time (min)[a]	% Specific release[b]	% Control release
Control	(no unlabeled P815)	52.4	100
0	0	35.9	69
0.1%	1 min	38.0	73
0.1%	3 min	38.5	74
0.3%	1 min	40.1	77
0.3%	3 min	45.5	87
0.9%	3 min	46.3	88
1.8%	3 min	45.5	87
3.6%	3 min	45.7	87

[a]Cells were fixed in phosphate-buffered saline (PBS), diluted 25-fold with PBS/30 mM glycine after 1 or 3 minutes and then washed immediately in PBS followed by EGTA/Mg^{++} medium.
[b]CTL-labeled P815 conjugates were formed by centrifugation. A 16-fold excess of unlabeled, fixed P815 was then added (final 2×10^4 ^{51}Cr-labeled P815/ml, E/T = 6:1), and the cells were again centrifuged to induce reversal. Standard deviations for quadruplicate samples were less than 1.5%; the spontaneous ^{51}Cr release was 14.3%.

The reversal measured here appears to be due to an exchange process whereby the CTL binds an unlabeled target cell and releases its ^{51}Cr-labeled target cell. Several results support this conclusion. Spontaneous release of bound P815 target cells in suspension was not readily detectable, but significant release was induced when unlabeled P815 target cells were added (Figs. 2, 3). No reversal was seen when ^{51}Cr-labeled target cells, rather than unlabeled P815, were added (not shown). Furthermore, when the initial conjugates were formed with unlabeled P815 and reversal was done using labeled P815, significant binding of the added labeled P815 was observed (data not shown).

The disparity observed between spleen cells and tumor cells therefore appears to relfect the CTL's ability to release a target cell that is weakly bound (spleen cell targets) in exchange for a more tightly bound (higher apparent affinity) tumor target cell. The factors responsible for this preferential binding of tumor cell targets, and for the ineffectiveness of purified plasma membranes in inducing reversal [9 and unpublished results], are unclear. The role played by receptor-H-2 interactions in holding together CTL–target cell conjugates is unknown. The possibility that CTL have a second, non-H-2-dependent system for maintaining strong adhesions with target cells has been suggested [16]. We are currently trying to resolve some of these questions.

ACKNOWLEDGMENTS

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